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Mutational Analysis of the *Bacillus subtilis* DegU Regulator and Its Phosphorylation by the DegS Protein Kinase

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The DegS-DegU protein kinase-response regulator pair controls the expression of genes encoding degradative enzymes as well as other cellular functions in *Bacillus subtilis*. Both proteins were purified. The DegS protein was autophosphorylated and shown to transfer its phosphate to the DegU protein. Phosphoryl transfer to the wild-type DegU protein present in crude extracts was shown by adding ^{32}P-labeled DegS to the reaction mixture. Under similar conditions, the modified proteins encoded by the *degU24* and *degU31* alleles presented a stronger phosphorylation signal compared with that of the wild-type DegU protein. This may suggest an increased phosphorylation of these modified proteins, responsible for the hyperproduction of degradative enzymes observed in the *degU24* and *degU31* mutants. However, the *degU32* allele, which also leads to hyperproduction of degradative enzymes, encodes a modified DegU response regulator which seems not to be phosphorylatable. The expression of the hyperproduction phenotype of the *degU32* mutant is still dependent on the presence of a functional DegS protein. DegS may therefore induce a conformational change of the *degU32*-encoded response regulator enabling this protein to stimulate degradative enzyme synthesis. Two alleles, *degU122* and *degU146*, both leading to deficiency of degradative enzyme synthesis, seem to encode phosphorylatable and nonphosphorylatable DegU proteins, respectively.

Matched pairs of regulatory proteins, designated modulators and response regulators, control a large number of bacterial adaptive responses. These include chemotaxis; control of expression of genes or sets of genes belonging to complex regulons, i.e., virulence genes of pathogenic bacteria; and developmental pathways such as sporulation or competence for DNA uptake (36). Six proteins of the modulator family, CheA (7, 39), NtrB (26), VirA (9, 13), EnvZ (1, 5, 10), PhoR (20), and KinA (28), have been shown to act as protein kinases, are autophosphorylated at a histidine residue, and catalyze the transfer of a phosphate group to their cognate response regulator (also called effector).

The modulator-response regulator pair DegS-DegU of *Bacillus subtilis* is involved in the control of expression of different cellular functions, including degradative enzyme synthesis, competence for DNA uptake, presence of flagella, and control of sporulation (16, 22, 29).

The corresponding genes, *degS* and *degU*, were initially defined by different classes of mutations (6, 15, 22) leading either to deficiency of degradative enzyme synthesis (designated *degS* or *degU* mutations) or to overproduction of degradative enzymes [designated *degS*(Hy) or *degU*(Hy) mutations].

The *degS* and *degU* genes are essential for the production of degradative enzymes, which include an intracellular protease and several enzymes which are secreted into the culture medium: levansucrase, proteases, α-amylase, β-glucanase(s), and xylanase. These enzymes are synthesized during the exponential growth phase (e.g., levansucrase) or during the early-stationary phase (e.g., proteases and α-amylase). Deletion or disruption of the *degU* gene abolished both degradative enzyme synthesis and genetic competence, indicating that this gene is essential for the expression of both cellular functions (15, 22, 29, 37). A strain from which the *degS* gene was deleted and in which the *degU* gene was expressed from the *degS-degU* operon promoter developed normal genetic competence, but the rate of degradative enzyme synthesis was reduced compared with that of the parental strain *B. subtilis* 168 (23).

As suggested earlier (22), DegS has protein kinase activity. This has now been confirmed by two independent reports (Mukai et al. [24] and this paper). Since a functional DegS protein is apparently required for degradative enzyme synthesis but not for competence, we proposed the hypothesis that the unphosphorylated form of the DegU response regulator may be required for genetic competence, while the phosphorylated form may be required for expression of genes encoding degradative enzymes (22). In agreement with this hypothesis is the observation that the *degU146* mutant is deficient for degradative enzyme synthesis but retains the wild-type level of competence. This mutation leads to the replacement of the aspartate residue at position 56 by asparagine in the DegU amino acid sequence. This aspartate residue is thought to represent the site of phosphorylation of the DegU protein, since aspartate residues at equivalent positions in the CheY and VirG response regulators were identified as the phosphoryl acceptor sites (12, 32). The *degS*(Hy) and *degU*(Hy) mutations may lead to increased phosphorylation of DegU. However, alternative hypotheses may be envisaged (see Results).

In contrast to genetic competence and degradative enzyme synthesis which may require a functional unphosphorylated or phosphorylated DegU response regulator, respectively, sporulation and flagellar synthesis can occur in the absence of the *degS-degU* regulatory system (22). However, control of these two processes is altered by *degS*(Hy) and *degU*(Hy) mutations, leading to the ability to sporulate in the presence of glucose and to loss of flagella.

In this paper, we describe the purification of the DegS protein kinase and show that the DegS protein is autophosphorylated and catalyzes the transfer of phosphate to the

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TABLE 1. Bacterial strains used in this study

Strain	Genotype[a]	Source or reference
Escherichia coli TG1	Δ(*lac-proAB*) *supE thi hsdD5* (F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ* ΔM15)	5a
Bacillus subtilis		
168	*trpC2*	Laboratory stock
1A510	*leuA8 arg-15 thrA5 recE4 stp*	27
QB136	*trpC2 leuA8 degU32*(Hy)	16
QB152	*trpC2 leuA8 degU31*(Hy)	Laboratory stock
QB256	*trpC2 hisA1 sacA321 degU122*	19
QB261	*sacA331 degU32*(Hy) *degU146*	Laboratory stock
QB269	*sacA331 degS220 degU32*(Hy)	16
QB315	*trpC2 leuA8 degU24*(Hy)	19
QB4224	*leuA8 arg-15 thrA5 recE4 stp*, carrying plasmid pBU16	15
QB4238	*trpC2* Δ(*degS degU*)::*aphA3*	22
QB4407	*trpC2 leuA8 degSΔE degU32*(Hy) *aphA3*	pBU128→QB136[b]
QB4414	*trpC2 degU146*	pMD406→168[b,c]

[a] *aphA3* indicates the *Streptococcus faecalis* kanamycin resistance gene (38).
[b] Arrows (→) indicate construction by transformation.
[c] For detailed description of construction, see Materials and Methods.

purified DegU response regulator. These findings were recently reported independently by Mukai and co-workers (24).

We were also able to perform transphosphorylation reactions by using crude extracts and purified DegS, thereby developing a rapid assay system to examine whether mutationally altered DegU response regulators could serve as phosphate acceptors for the DegS protein kinase. This assay system enabled us to identify two classes of *degU* mutations. The mutant proteins either presented a stronger phosphorylation signal or did not seem to be phosphorylatable under the conditions tested.

MATERIALS AND METHODS

Strains. Bacterial strains used in this study are shown in Table 1. *Escherichia coli* K-12 strain TG1 carrying plasmid pMD402 was grown in Luria broth containing ampicillin (100 μg/ml). *B. subtilis* strains were grown in Penassay antibiotic medium 3 (Difco Laboratories, Detroit, Mich.). Strains QB4224 and QB4407 were grown in the same medium with 5 μg of chloramphenicol or kanamycin per ml, respectively.

Transformation of *E. coli* and *B. subtilis* was carried out as previously described (3, 15, 35).

DNA manipulations. Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs, Inc., Beverly, Mass., and used as recommended. DNA fragments were purified from agarose gels by using a Gene Clean kit (Bio 101, La Jolla, Calif.). Plasmids were prepared by using a Qiagen kit (Diagen GmbH, Düsseldorf, Federal Republic of Germany). All other DNA standard procedures were carried out as described by Sambrook et al. (31). Polymerase chain reactions (25, 30) were carried out as previously described (23). DNA sequences were determined by using the dideoxy-chain termination method (33), modified T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, Ohio), and single-stranded polymerase chain reaction products produced through asymmetric amplification (11, 34).

Plasmids. Overproduction of the DegS protein was obtained by constructing plasmid pMD402, in which the *degS*-coding sequence is expressed under control of the strong *B. subtilis degQ36* promoter (2, 23, 40). This plasmid was constructed in several steps. A 1.5-kb *Xba*I-*Eco*RV fragment from plasmid pBU100 (15), which contains the *degS*-coding sequence, including its ribosome-binding site, was cloned between the *Xba*I and *Hin*cII sites of pUC18, yielding plasmid pMD400. Between the *Eco*RI and *Sma*I sites preceding the *degS*-coding sequence in pMD400 was placed a 218-bp fragment carrying the *degQ36* promoter, which yielded plasmid pMD402. This fragment extends from position −186 to +32 with respect to the transcriptional start site of the *degQ36* promoter. It was synthesized by polymerase chain reaction amplification with a *degQ36*-containing plasmid (pBQ109) as a template and specific primers creating *Eco*RI and *Sma*I sites upstream and downstream from the *degQ36* promoter, respectively (23).

An in-frame deletion eliminating 60 bp of the *degS*-coding sequence between the *Hin*cII and *Pvu*II sites was constructed in the following manner. A 2.3-kb *Eco*RI-*Hin*cII fragment from plasmid pBU126 (23) containing the *aphA3* kanamycin resistance determinant upstream from the *degS* gene and a 980-bp *Pvu*II-*Bst*BI fragment from plasmid pBU100 were cloned between the *Eco*RI and *Cla*I sites of the pJH101 plasmid vector (4a), to give plasmid pBU128. The deleted gene, designated *degSΔE*, in which 20 codons of the DNA region coding for the amino-terminal part of the DegS protein are missing, was transferred to the expression vector by replacing the 608-bp *Xba*I-*Afl*II fragment of pMD402 with the 548-bp *Xba*I-*Afl*II fragment of pBU128, giving plasmid pMD404.

Plasmid pBU16 used for overproduction of the wild-type DegU protein carries both wild-type *degS* and *degU* genes (15).

A strain carrying the *degU146* mutation alone was constructed from strain QB261 which carries both the *degU32* and *degU146* mutations (Table 1). Plasmid pBU16 was introduced into strain QB261 by transformation. Allelic exchange occurred between the wild-type plasmid-borne *degU* allele and the deficient chromosomal allele. Segregants deficient for levansucrase production were isolated. Plasmid pBU139, carrying both the *degU32* and *degU146* mutations, was isolated from one of these clones. From plasmid pBU139 we constructed a plasmid containing the *degU146* mutation alone. We took advantage of the following: (i) single *Bcl*I and *Sst*I sites are present in both plasmids, and (ii) the *Bcl*I site is located between the *degU32* and *degU146* mutations in plasmid pBU139. The large *Bcl*I-*Sst*I fragment of plasmid pBU16 was ligated with the small *Bcl*I-*Sst*I fragment of pBU139 containing the *degU146* mutation alone. The ligation mixture was introduced into *B. subtilis* 1A510, and chloramphenicol-resistant transformants were selected as previously described (15), yielding plasmid pMD406. DNA sequencing with plasmid pMD406 as a template showed that the *degU146* mutation alone was present. Introduction of plasmid pMD406 into *B. subtilis* 168 by transformation, followed by substitution of the chromosomal allele with the deficient *degU146* allele and loss of the plasmid, allowed the isolation of strain QB4414 carrying the *degU146* mutation alone (Table 1).

Gel electrophoresis. Crude extracts of sodium dodecyl

sulfate (SDS)-lysed *E. coli* cells were prepared by the method of Silhavy et al. (35) for rapid analysis of the presence of the DegS protein. SDS-polyacrylamide gel electrophoresis (PAGE) on 12% acrylamide gels was performed by the method of Laemmli (17) by using a minigel system (Bio-Rad Laboratories, Richmond, Calif.). Before application to the gel, ^{32}P-labeled protein samples were heated at 55°C for 10 min and nonlabeled protein samples were heated at 100°C for 10 min. Molecular size reference markers were obtained from Bio-Rad. After SDS-PAGE, nonlabeled proteins were stained with Coomassie blue. The polyacrylamide gels containing ^{32}P-labeled proteins were washed once for 5 min in destaining solution (20% ethanol–7.5% acetic acid–2% glycerol [vol/vol]), dried on Whatman 3MM paper at 60°C, and exposed to Kodak X-Omat-S film.

Purification of DegS. Three liters of *E. coli* TG1 carrying plasmid pMD402 were grown at 37°C to the stationary phase (optical density at 600 nm = 3). Cell extracts were prepared as described by Perego et al. (28) with minor modifications. Cultures were centrifuged at 5,000 × *g* for 10 min, washed twice with 300 ml of buffer A (25 mM Tris-HCl [pH 8.0], 10 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 7 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride), and resuspended in 40 ml of the same buffer. The cells were treated with lysozyme (100 μg/ml) for 30 min at 4°C and sonicated as previously described (28). After centrifugation for 1 h at 40,000 × *g* in a Sorvall SS34 rotor, the supernatant was removed and the pellet was resuspended in 4 ml of buffer A and stored at −20°C. An aliquot (30 μl) of this suspension was diluted to 1 ml with buffer B (50 mM Tris-HCl [pH 8.0], 50 mM potassium chloride, 1 mM EDTA, 1 mM dithiothreitol) containing 6 M urea and centrifuged at 5,000 × *g* for 10 min. The supernatant was collected and incubated on ice for 5 min with an equal volume (1 ml) of settled suspension of QAE-Sephadex A-50 (Pharmacia) which was previously equilibrated with buffer B. Every 5 min, 1 ml of buffer B was added, and the suspension was carefully mixed by shaking and was incubated on ice. This operation was repeated until the protein solution was diluted 10-fold (10 ml, total volume). The QAE-Sephadex beads containing the adsorbed proteins were centrifuged at 5,000 × *g* for 10 min, and the proteins were eluted stepwise by buffer B containing increasing concentrations of potassium chloride. This was performed by resuspension of the beads in buffer B containing 100 mM potassium chloride (1 ml of buffer per ml of settled beads), which was followed by incubation at 4°C with shaking for 5 min. After centrifugation, the supernatant was kept and the proteins were subsequently eluted as described above with buffer B containing 200 mM, 500 mM, 1 M, or 2 M potassium chloride. The different eluted samples were analyzed by SDS-PAGE, and the purified DegS protein was dialyzed against buffer B containing 200 mM potassium chloride to remove all urea.

By using the same method, DegSΔE was purified from *E. coli* TG1 carrying plasmid pMD404.

***B. subtilis* crude protein extracts.** The indicated *B. subtilis* strains were grown in Penassay broth to an optical density at 600 nm of 1.0, centrifuged at 10,000 × *g* in a Sorvall GS-3 rotor for 10 min, and resuspended in 1/40 culture volume of buffer C (10 mM potassium phosphate [pH 7.5], 1 mM EDTA, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride). The cells were sonicated for 4 or 5 cycles of 1-min bursts separated by 1-min rests in a Branson sonifier model B-12. Cell debris were removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was used as a crude protein extract.

Purification of DegU. Strain QB4224 was grown in Penassay broth, and a crude extract was prepared as described above. With gentle stirring at 4°C, a 10% (wt/vol) streptomycin sulfate solution was slowly added to the extract to give a final concentration of 1%. The incubation was continued without stirring for 15 min. The nucleic acid precipitate was removed by centrifugation at 10,000 × *g* for 10 min. With gentle stirring at 4°C, solid ammonium sulfate was slowly added to 45% saturation. When the ammonium sulfate was dissolved, incubation was continued for 15 min without stirring. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min and dissolved in a minimal volume of buffer B. After dialysis against buffer B with one change, a 2-ml sample containing 40 mg of protein as determined by using a protein assay (Bio-Rad) was loaded on an Ultrogel AcA 44 column (58 by 1.2 cm; IBF-Biotechnics). Fractions of 1 ml were collected and analyzed by SDS-PAGE for the presence of a prominent band of about 30 kDa corresponding to the DegU protein. The DegU-enriched fractions were pooled and dialyzed against buffer D (10 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol) with one change. A sample containing 1.2 mg of protein was loaded on an Affi-Gel Cibacron Blue column (1.8 by 0.9 cm; Bio-Rad) in a 2-ml syringe. The column was washed with 6 ml of buffer D, and 0.5-ml fractions were collected at a rate of less than 10 ml/h. Pure DegU protein was eluted by buffer D containing 200 mM NaCl.

Phosphorylation of DegS. The in vitro phosphorylation assay was carried out by the methods of Keener and Kustu (14) and Perego et al. (28) with some modifications. Phosphorylation reaction mixture (50 μl) contained 20 μg of purified DegS protein in buffer E (100 mM Tris-HCl [pH 8.0], 200 mM KCl, 4 mM $MgCl_2$, 4 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 5% [vol/vol] glycerol). The reaction was initiated by addition of 30 μCi of [γ-^{32}P]ATP diluted with nonlabeled ATP to give a final concentration of 2.5 μM. [γ-^{32}P]ATP was obtained from Amersham International, Buckinghamshire, England. The reaction mixture was incubated at 25°C for the indicated times and was stopped by adding 0.1 volume of a solution containing 10% SDS–0.02% bromophenol blue and immediately frozen at −20°C. Samples were analyzed by SDS–12% PAGE and autoradiography.

For the determination of the apparent K_m, purified DegS protein (20 μg) was incubated in reaction buffer E (50 μl) containing the indicated concentrations of [γ-^{32}P]ATP. Aliquots (5 μl; 2 μg of protein) were applied to nitrocellulose filters previously soaked in 5% trichloroacetic acid, which were immediately washed (for less than 20 s) with 5% trichloroacetic acid. After the filter was dried, radioactivity was assayed by scintillation counting. Initial rates of incorporation were determined from a linear regression analysis of these data.

Isolation of phosphorylated ^{32}P-DegS. Phosphorylated ^{32}P-DegS was separated from ATP in the following manner: a 200-μl reaction mixture containing 80 μg of ^{32}P-DegS–90 μCi of [γ-^{32}P]ATP with a final concentration of 2.5 μM nonlabeled ATP was precipitated by adding ammonium sulfate to 70% saturation in the presence of 200 μg of carrier bovine serum albumin (Miles; 3× crystallized). The suspension was incubated for 20 min at 0°C and centrifuged for 5 min at 12,000 × *g* at 4°C. The precipitate was washed with 70% saturated ammonium sulfate in buffer B, centrifuged again, and resuspended in 50 μl of buffer B containing 200 mM

potassium chloride. Remaining ammonium sulfate and [γ-^{32}P]ATP were eliminated through dialysis by placing the reaction mixture on a Millipore VMWP filter (0.05 μm pore size) floating on 20 ml of buffer B containing 200 mM KCl for 3 h at 0°C. The dialysis buffer was changed once after 90 min of incubation. The ^{32}P-DegS was essentially free of [γ-^{32}P]ATP as verified by SDS-PAGE and autoradiography.

Phosphorylation of DegU. DegS was incubated for 2 min in buffer E containing [γ-^{32}P]ATP to produce autophosphorylated ^{32}P-labeled DegS as described above. The reaction mixture (10 μl) was added to 90 μl of buffer E containing nonlabeled ATP (0.6 mM, final concentration) and 5.4 μg of purified DegU. After incubation at 25°C for the indicated times, 20-μl aliquots in which the transphosphorylation reaction was stopped by adding 3 μl of 10% SDS–0.02% bromophenol blue were removed.

Transphosphorylation reactions with crude extracts were carried out as follows: 0.5 μg of ^{32}P-labeled autophosphorylated DegS protein prepared as described above was mixed with nonradioactive ATP (0.6 mM, final concentration) and immediately added to 17 μl of crude extract in buffer E containing 400 μg of total protein and incubated at 25°C for the indicated times. The reaction was stopped and analyzed as described above.

RESULTS

Overproduction and purification of DegS and DegSΔE proteins. Preliminary experiments to overproduce the DegS protein in either *E. coli* or *B. subtilis* were carried out. Two different promoters were used: the *E. coli tac* promoter and the *B. subtilis degQ36* promoter (18, 40). From these data we concluded that the *degQ36* promoter led to optimal expression of the *degS* gene in *E. coli* by using plasmid pMD402 (results not shown). Expression from the *degQ36* promoter increased in *E. coli* cells after the end of the exponential growth phase as earlier observed in the case of *B. subtilis* cells (40) and was found to be optimal at an incubation temperature of 37°C. The conditions of DegS overproduction were thus established: cultures of *E. coli* containing plasmid pMD402 or pMD404 (see Materials and Methods) were grown overnight at 37°C in Luria broth for production of the wild-type DegS protein and the modified DegSΔE protein, respectively. This modified protein is encoded by the *degSΔE* gene, which was constructed from the wild-type *degS* gene by deleting 20 codons corresponding to the amino-terminal part of the DegS protein. The use of this modified protein will be discussed below.

After sonication of the cells and centrifugation, overproduced DegS and DegSΔE proteins were found as inclusion bodies in the pellet. Analysis by gel electrophoresis showed major bands of approximately 43 kDa molecular mass, and densitometer scanning of the Coomassie blue-stained polyacrylamide gels indicated that DegS and DegSΔE made up 45 and 35% of the total protein in the pellet, respectively (Fig. 1A). These two proteins were purified by solubilizing the proteins with 6 M urea and gradual dilution of the urea-treated pellet with buffer B containing QAE-Sephadex A-50 (8). During this process, DegS and DegSΔE were both renatured and adsorbed to the QAE-Sephadex A-50 beads, which were then centrifuged. DegS and DegSΔE were eluted from the beads with buffer B containing stepwise increasing concentrations of potassium chloride (see Materials and Methods). Using buffer B containing 200 and 100 mM potassium chloride, the DegS and DegSΔE proteins were eluted with more than 90 and 85% purity, respectively (Fig. 1A). To remove urea from the sample, the DegS and DegSΔE proteins were dialyzed against buffer B containing 200 mM potassium chloride. Under these conditions, the proteins remained soluble. Dialysis against buffer B containing less than 100 mM potassium chloride led to precipitation of these proteins.

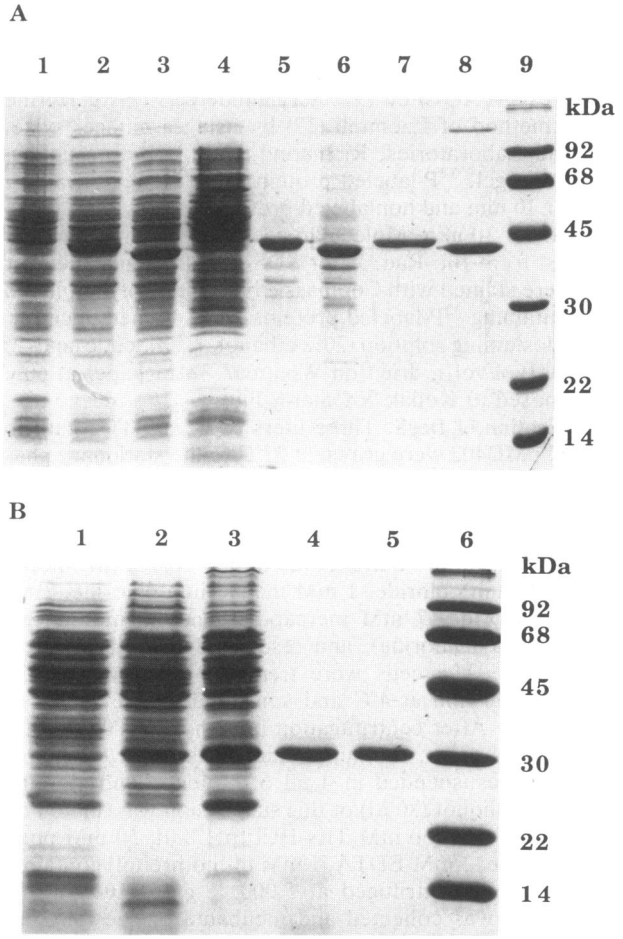

FIG. 1. (A) SDS-PAGE showing purification of DegS and DegSΔE. Lane 1, Extract from SDS-lysed cells of *E. coli* TG1; lane 2, extract from SDS-lysed cells of strain TG1 containing plasmid pMD402; lane 3, extract from SDS-lysed cells of strain TG1 (pMD404); lane 4, supernatant obtained after sonication of TG1 (pMD402); lane 5, pellet obtained after sonication of TG1(pMD402); lane 6, pellet obtained after sonication of TG1(pMD404); lane 7, purified DegS protein; lane 8, purified DegSΔE protein; lane 9, molecular mass standards with sizes indicated. (B) SDS-PAGE showing purification of DegU. Lane 1, Extract from *B. subtilis* 1A510 containing pHV1431d; lane 2, extract from strain 1A510 containing pBU16 (strain QB4224); lane 3, ammonium sulfate fraction (0 to 45% saturation) of extract from strain QB4224; lane 4, pooled fraction after AcA 44 chromatography; lane 5, purified DegU protein after Affi-Gel Cibacron Blue chromatography; lane 6, molecular mass standards with sizes indicated. For details see Materials and Methods.

Overproduction and purification of DegU. The wild-type *degU* gene was cloned in *B. subtilis* by using the high-copy-number plasmid vector pHV1431d (15). *B. subtilis* 1A510 containing the recombinant plasmid pBU16 overproduced the DegU protein, giving a major band on a Coomassie blue-stained polyacrylamide gel of approximately 30 kDa molecular mass. After elimination of nucleic acids by strep-

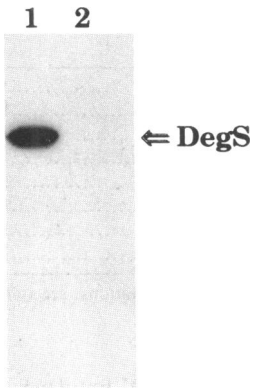

FIG. 2. Autoradiogram after SDS-PAGE, showing autophosphorylation of the DegS protein. Lane 1, Autophosphorylated DegS; lane 2, no autophosphorylation observed by using the DegSΔE protein. The phosphorylation reactions were carried out for 2 min at 25°C as described in Materials and Methods. The film was exposed for 30 min at room temperature.

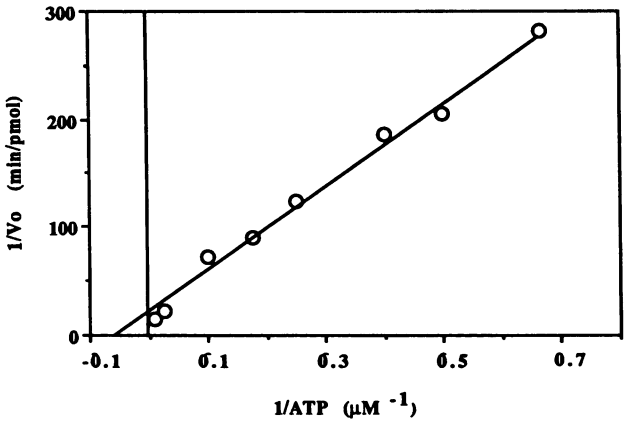

FIG. 3. Initial rate of DegS phosphorylation (V_0, in picomoles per minute) as a function of the ATP concentration (in micromolar). Aliquots (5 μl) were taken after 10, 30, and 60 s and 2, 4, and 10 min, applied to nitrocellulose, and analyzed for phosphorylation as described in Materials and Methods. The data are presented in a double reciprocal plot. An apparent K_m of 20 μM was determined by linear regression analysis.

tomycin sulfate precipitation, the DegU protein was purified by ammonium sulfate fractionation, gel filtration, and Affi-GelCibacron Blue column chromatography (Fig. 1B). As was previously described for the CheY response regulator (21), the DegU protein also binds to Cibacron Blue. The DegU protein, in contrast with DegS, remains soluble in low-salt buffers (10 mM Tris-HCl [pH 7.5]).

Autophosphorylation of DegS. On the basis of amino acid sequence similarities, we proposed earlier that DegS may be a member of a family of protein kinases which are phosphorylated at histidine residues (15, 22, 36). We show here that the DegS protein has autophosphorylation activity; after 2 min of incubation of purified DegS in the presence of [γ-^{32}P]ATP and divalent cations (see Materials and Methods), the protein in the reaction mixture was labeled as shown by SDS–12% PAGE followed by autoradiography (Fig. 2). By analogy with other histidine protein kinases it is likely that a conserved histidine residue present in DegS (His-189) is the phosphoacceptor site.

In several cases, it has been shown that a truncated histidine protein kinase containing the carboxy-terminal domain alone possesses autophosphorylation activity (EnvZ, PhoR, and VirA) (1, 5, 9, 10, 13, 20). This is apparently not the case for DegS, since a truncated protein, DegSΔE (from which amino acids 23 to 42 were removed by deletion) lost autophosphorylation activity completely (Fig. 2).

Initial rates of phosphorylation were determined for DegS as a function of ATP concentration to determine the apparent K_m, which was found to be about 20 μM ATP (Fig. 3). DegS has a stronger affinity for ATP than, for instance, the *Salmonella typhimurium* CheA protein kinase (K_m = 290 μM ATP) (39) (see Discussion).

Phospho-DegS transfers its phosphate to the DegU protein. In other well-studied protein kinase-response regulator pairs, i.e., NtrB-NtrC, CheA-CheY, EnvZ-OmpR, PhoR-PhoB, and VirA-VirG, the phosphorylated protein kinases rapidly transfer their phosphate groups to the response regulators. Phosphorylation of DegU by DegS was shown in a two-step reaction, as follows. Purified DegS was incubated in the presence of [γ-^{32}P]ATP and divalent cations (see Materials and Methods). The reaction mixture, containing phosphorylated ^{32}P-DegS, was then added to the purified DegU protein in the presence of an excess of nonlabeled ATP [0.6 mM]. ^{32}P was transferred from DegS to DegU, as shown by SDS-PAGE followed by autoradiography (Fig. 4). These results demonstrate that the DegU protein is phosphorylated in the presence of the DegS protein kinase. The phosphorylated DegU protein appears to be quite stable, since the phospho-DegU signal did not decrease until after 20 min of incubation (Fig. 4).

Incubation of purified DegU alone in the presence of [γ-^{32}P]ATP did not lead to phosphorylation (data not shown). In addition, it is unlikely that the DegU protein is autophosphorylated in the presence of the DegS protein, since ^{32}P-DegS which is essentially free of [γ-^{32}P]ATP (see Materials and Methods) also catalyzes rapid phosphoryl transfer to the DegU protein (data not shown).

Effects of *degU* mutations on phosphorylation. Since the conditions of phosphoryl transfer from DegS to DegU were

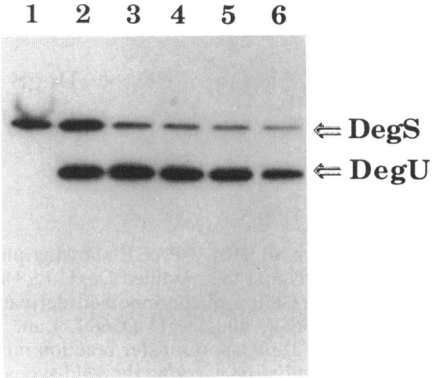

FIG. 4. Autoradiogram after SDS-PAGE, showing time dependence of phosphoryl group transfer from purified DegS to purified DegU. Lane 1, Autophosphorylated DegS alone. The phosphotransfer reactions were carried out in a two-step reaction as described in Materials and Methods and incubated at room temperature for 1 min (lane 2), 5 min (lane 3), 10 min (lane 4), 15 min (lane 5), and 20 min (lane 6). The film was exposed with an intensifying screen for 2 h at room temperature.

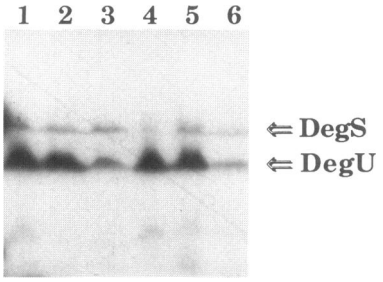

FIG. 5. Autoradiogram after SDS-PAGE showing phosphorylation of the modified DegU V131/L, DegU T98/I, and the wild-type DegU proteins in crude extracts by the purified DegS protein kinase. Crude extracts from strain QB152 carrying the *degU31*(Hy) mutant (lanes 1 and 4), strain QB315 carrying the *degU24*(Hy) mutant (lanes 2 and 5), or the wild-type strain *B. subtilis* 168 (lanes 3 and 6) were used in two-step transphosphorylation reactions as described in Materials and Methods. Reaction mixtures were incubated at room temperature for 1 min (lanes 1, 2, and 3) and 5 min (lanes 4, 5, and 6). The film was exposed for 48 h at −70°C by using an intensifying screen.

established by using purified proteins, we asked whether this reaction was sensitive enough to allow detection of phosphorylation of DegU in crude extracts. DegS was autophosphorylated by incubation with [γ-^{32}P]ATP. In a second step, crude extract was added to the reaction mixture in the presence of an excess of nonradioactive ATP (0.6 mM) to minimize radioactive labeling of other proteins such as protein kinases or response regulators other than DegS or DegU, which could also be present in the crude extract.

After incubation of ^{32}P-DegS with a crude extract from *B. subtilis* 168 containing the wild-type DegU protein and analysis by SDS-PAGE and autoradiography, the DegU protein is labeled (Fig. 5 and 6). As observed for other systems, phosphate transfer is a rapid process (12, 20, 39), since the radioactive labeling of DegU occurred as early as 10 s from the start of incubation at 25°C (data not shown). Incubation of ^{32}P-DegS with crude extract prepared from

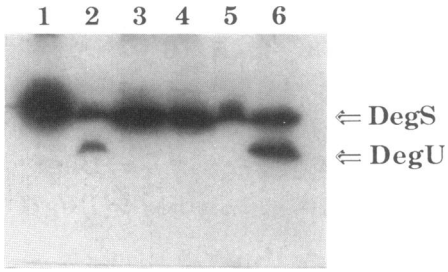

FIG. 6. Autoradiogram after SDS-PAGE showing phosphorylation of the wild-type DegU and the modified DegU R184/C proteins and absence of phosphorylation of the modified derivatives DegU H12/L, DegU H12/L D56/N, and DegU D56/N. Lane 1, Purified DegS protein alone; lane 2, phosphotransfer reaction mixtures with a cell extract from *B. subtilis* 168 carrying the wild-type *degU* gene; lane 3, extract from *B. subtilis* QB136 carrying the *degU32*(Hy) mutation; lane 4, extract from the *B. subtilis* QB261 double mutant *degU32*(Hy) *degU146*; lane 5, extract from *B. subtilis* QB4414 carrying the *degU146* mutation; lane 6, extract from strain QB256 carrying the *degU122* mutation. The two-step transphosphorylation reaction was carried out as described in Materials and Methods. Reaction mixtures were incubated at room temperature for 2 min. The film was exposed for 48 h at −70°C with an intensifying screen.

TABLE 2. Mutant alleles and corresponding modified proteins

Allele[a]	Modified protein
degS220	DegS A193/V
degSΔE	DegS Δ(D23-Q42)
degU24	DegU T98/I
degU31	DegU V131/L
degU32	DegU H12/L
degU32 degU146	DegU H12/L D56/N
degU122	DegU R184/C
degU146	DegU D56/N

[a] For detailed description of the mutations, see reference 22.

strain QB4238 which is deleted for *degS* and *degU* showed no additional labeled protein bands (data not shown).

In this way, we developed a rapid screening method allowing us to analyze whether mutationally altered DegU response regulators could serve as phosphoacceptors in transphosphorylation reactions.

We previously suggested (22) that unphosphorylated DegU may be needed for the expression of genes involved in development of genetic competence of *B. subtilis*, whereas phosphorylated DegU may be required for the expression of genes encoding degradative enzymes. The *degU*(Hy) mutations would lead to increased rates of phosphorylation or decreased rates of dephosphorylation of the DegU protein. We examined DegU phosphorylation in crude extracts prepared from two of these mutants, *degU24*(Hy) and *degU31*(Hy), and the wild-type strain 168, which was included as a control. To these extracts was added ^{32}P-DegS, which was followed by incubation in the presence of nonradioactive ATP, and samples were analyzed by SDS-PAGE and autoradiography as described in Materials and Methods. The DegU proteins encoded by the *degU24* and *degU31* alleles present threonine to isoleucine and valine to leucine changes at positions 98 and 131 and are designated DegU T98/I and DegU V131/L, respectively (Table 2). The DegU T98/I and DegU V131/L proteins showed a stronger phosphorylation signal compared with that of the wild-type DegU protein (Fig. 5) (see Discussion).

The *degU146* mutation in strain QB261 was identified as a mutation leading to the suppression of the Hy phenotype (hyperproduction of degradative enzymes) of a *degU32* mutant. Because of the presence of both the *degU32* and *degU146* mutations in this strain, the modified protein contained two changes and is designated DegU H12/L D56/N (Table 2). This modified protein could not be phosphorylated (Fig. 6). The protein containing the D56/N change alone, produced by strain QB4414 (constructed as described in Materials and Methods), also could not be phosphorylated (Fig. 6). Our interpretation was that this protein had lost the putative site of phosphorylation (D56; see Introduction). However, we cannot exclude the possibility that the modified protein may be unstable in the crude extract. It also seemed of interest to study phosphorylation of the *degU32*-encoded protein containing the H12/L change alone. Our initial prediction was that *degU32*(Hy) encoded a hyperphosphorylated DegU protein as described above for *degU24*(Hy)- and *degU31*(Hy)-encoded proteins. Surprisingly, the *degU32*(Hy)-encoded protein could not be phosphorylated under the conditions used (Fig. 6) (see Discussion). This was the result of three experiments using independently prepared extracts.

The absence of phosphorylation of the *degU32*-encoded protein might be the consequence of strong DegU phos-

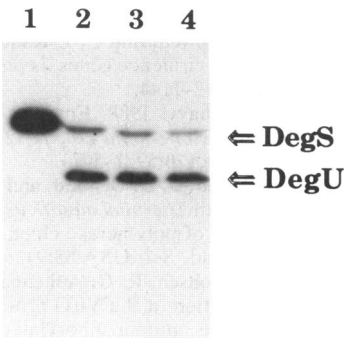

FIG. 7. Control experiment using purified wild-type DegU in the presence of crude extracts from *B. subtilis* strains. Lane 1, Purified and autophosphorylated DegS. Phosphorylation of purified DegU in the presence of crude extracts from strains QB261 (*degU32 degU146*), 168 (wild type), and QB136 (*degU32*) is shown in lanes 2, 3, and 4, respectively. The reaction mixtures were incubated in the presence of ^{32}P-DegS for 2 min. The film was exposed for 16 h at −70°C with an intensifying screen.

phatase activity in the extract prepared from the *degU32* mutant. This possibility seemed unlikely, since similar rates of transphosphorylation were observed in reaction mixtures containing purified DegS and DegU proteins in the presence of extracts prepared from strain QB136 or QB261 or the wild-type strain 168 (Fig. 7).

The mutations presented above correspond to alterations of the amino-terminal receiver domain of the DegU protein leading to alterations of the rate of DegU phosphorylation (22; this paper). It seemed interesting to examine the phosphorylation of a DegU protein modified in its carboxy-terminal domain. We chose such a modified protein, DegU R184/C, encoded by the *degU122* allele, which leads to deficiency of degradative enzyme synthesis (22). We found that the DegU R184/C protein was phosphorylated (Fig. 6), indicating that the *degU122* mutation leads to degradative enzyme production deficiency without impairing DegU phosphorylation.

DISCUSSION

The DegS-DegU two-component regulatory system affects the synthesis of degradative enzymes and competence of DNA uptake as well as other cellular functions in *B. subtilis* (see Introduction).

We showed in this paper that the DegS protein kinase phosphorylates the pleiotropic DegU regulatory protein of *B. subtilis* by using purified preparations of both proteins. This result was reported independently by Mukai et al. (24).

We suggested earlier (22) that different forms of the DegU protein affect the expression of target genes as follows: (i) phosphorylated DegU is required for the production of a class of degradative enzymes, and (ii) unphosphorylated DegU is, on the contrary, required for the expression of competence genes but does not increase the expression of genes encoding degradative enzymes.

We could demonstrate phosphorylation of the wild-type DegU protein and modified regulators DegU T98/I and DegU V131/L in extracts from strain 168 and the *degU24*(Hy) and *degU31*(Hy) mutants, respectively. This was performed by showing phosphoryl transfer to the DegU proteins after adding ^{32}P-DegS to these extracts. Under identical conditions, the proteins encoded by the *degU24* and *degU31* mutants showed a stronger phosphorylation signal than the wild-type DegU protein. We favor the hypothesis that this is the consequence of differences in DegU protein activity reflecting better phosphotransfer or a longer half-life of phosphorylation of the mutant proteins. However, we cannot exclude differences in *degU* synthesis or protein stability between the wild-type strain and the mutants. Further work involving purification of these modified proteins will allow us to distinguish between these hypotheses. We also identified a modified DegU protein, DegU H12/L, encoded by the *degU32*(Hy) allele, which is not phosphorylated by DegS under the conditions used but still leads to overproduction of degradative enzymes and deficiency of genetic competence. However, absence of phosphorylation could also be the consequence of one of the following artifacts: (i) the QB136 extract might contain a DegU protein which is stably and completely phosphorylated, preventing further incorporation of ^{32}P-labeled phosphoryl groups; and (ii) the *degU32*-encoded protein could be unstable in the extracts prepared from strain QB136.

The first hypothesis is unlikely, since we found that the Hy phenotype of a *degU32* mutant could be suppressed by one of the following mutations in DegS: a deletion within the *degS*-coding sequence (*degSΔE*) or a *degS220* missense mutation (22; data not shown). Even in extracts from the double *degU32 degSΔE* and *degU32 degS220* mutants (strains QB4407 and QB269, respectively), no phosphorylation of the DegU H12/L protein by the added wild-type DegS protein could be demonstrated (data not shown). This observation may suggest that the DegU H12/L protein encoded by the *degU32* allele may not be phosphorylated and therefore may not depend on the protein kinase activity of DegS to stimulate degradative enzyme synthesis. If this were true, some other unidentified activity of the DegS protein could be required to enable the DegU H12/L protein to stimulate degradative enzyme synthesis, since strains containing a *degU32*(Hy) mutation and either one of two suppressor mutations, *degSΔE* (corresponding to an internal deletion within the *degS*-coding sequence) or the *degS220* missense mutation (Table 2), are deficient for degradative enzyme synthesis (22; data not shown).

These experiments were carried out by using crude preparations of mutationally altered DegU proteins and need to be confirmed by using purified proteins. However, our data seem to be consistent with the following conclusions.

(i) Phosphorylation of the wild-type DegU protein is required, probably through a transient conformational change of the DegU protein, for its activity as a transcriptional regulator of genes encoding degradative enzymes. A similar hypothesis was also proposed in the case of the VirA-VirG two-component system (12).

(ii) The stronger phosphorylation signal of the mutationally altered DegU T98/I and DegU V131/L proteins (encoded by the *degU24* and *degU31* alleles, respectively) could correspond to increased phosphorylation, leading to hyperproduction of degradative enzymes and lowered competence.

(iii) The DegU H12/L protein (encoded by *degU32*) may not be phosphorylated but may still require DegS activity to adopt a conformation allowing increase of degradative enzyme synthesis and decrease of genetic competence.

(iv) The DegU D56/N protein (encoded by *degU146*) leads to deficiency of degradative enzyme synthesis and a competence level comparable with that of the wild-type strain *B. subtilis* 168. This modified protein is thought to have lost the putative site of phosphorylation (D56). Our data are indeed

consistent with this hypothesis, since this protein could not be phosphorylated under the conditions used.

(v) The *degU122* mutation, modifying the carboxy-terminal domain of DegU, leads to deficiency of degradative enzyme synthesis but still allows phosphorylation of the encoded protein DegU R184/C.

The DegS protein may possess an activity distinct from its DegU protein kinase activity, allowing DegU H12/L to stimulate degradative enzyme synthesis. Such an activity may correspond to DegS-DegU protein contact, which may or may not require autophosphorylation of the DegS protein.

We showed that the apparent K_m of DegS (20 μM) is closer to the values obtained for eukaryotic protein kinases (e.g., the $p54^{src}$ oncogene with an apparent K_m of 30 μM) (4) than to those of other prokaryotic protein kinases (e.g., CheA of *S. typhimurium* with an apparent K_m of 290 μM) (39). This may reflect the amino acid sequence similarities found between the putative DegS ATP-binding site and the ATP-binding sites identified in eukaryotic protein kinases (22).

Future work will involve purification of modified DegS and DegU proteins in order to confirm data obtained by using crude extracts. It will be interesting to study how DegS may induce a putative conformational change of the DegU H12/L protein.

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